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A Comparison of TSPY Genes From Y-Chromosomal DNA of the Great Apes and Humans: Sequence, Evolution, and Phylogeny

HEUI-SOO KIM AND OSAMU TAKENAKA
Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Inuyama, Aichi 484, Japan

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ABSTRACT The genes for testis-specific protein Y (TSPY) were sequenced from chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), and baboon (*Papio hamadryas*). The sequences were compared with each other and with the published human sequence. Substitutions were detected at 144 of the 755 nucleotide positions compared. In overviewing five sequences, one deletion in human, four successive nucleotide insertions in orangutan, and seven deletions/insertions in baboon sequence were noted. The present sequences differed from that of human by 1.9% (chimpanzee), 4.0% (gorilla), 8.2% (orangutan), and 16.8% (baboon), respectively. The phylogenetic tree constructed by the neighbor-joining method suggests that human and chimpanzee are more closely related to each other than either of them is to gorilla, and this result is also supported by maximum likelihood and strict consensus maximum parsimony trees. The number of nucleotide substitutions per site between human and chimpanzee, gorilla, and orangutan for TSPY intron were 0.024, 0.048, and 0.094, respectively. The rates of nucleotide substitutions per site per year were higher in the TSPY intron than in the TSPY exon, and higher in the TSPY intron than in the ZFY (Zinc Finger Y) intron in human and apes.

The molecular evolution of Y-linked DNA sequences is of special interest because of their unique mode of inheritance (Lundrigan and Tucker, 1994). The Y-specific gene should be useful for the reconstruction of genetic and evolutionary history because of nonrecombining along most of its length and paternal inheritance (Dorit et al., 1995). The sequence divergence of the Y-chromosomal DNA among related genera and species can be useful in refining evolutionary classification and phylogenetic inference (Rasheed et al., 1991). Ellis et al. (1990), based on PABY (pseudoautosomal boundary Y) analyses, have human and chimpanzee as closest relatives to each other, followed by gorilla and then orangutan. However, the pseudoautosomal boundary region indicated that different phylogenies are supported by the pattern of species-level differentiation on the X vs. the Y chromosome. Comparative analyses of DNA sequences of β-globin (Bailey et al., 1992), mitochondrial DNA (Ruvolo, 1994; Horai et al., 1995), and variations of ribosomal DNA spacers using 12 restriction enzymes (Suzuki et al., 1994) support a closer relationship between human and chimpanzee.

TSPY (testis-specific protein Y), testis-specific gene (Weissenbach et al., 1989), was

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Address reprint requests to Osamu Takenaka, Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Inuyama, Aichi 484, Japan.

originally identified in an attempt to detect transcribed DNA sequences specific for the Y-chromosome. Analysis of the human cDNA clone pJ923 and subsequent comparison with genomic DNA result in the intronless gene (Arnemann et al., 1991). From PCR (polymerase chain reaction) and RT-PCR analyses (reverse transcription–PCR), however, the TSPY (Y-231) structural gene encompasses approximately 2.7 kb of genomic sequence and contains six exons (Zhang et al., 1992). The corresponding 1.3 kb cDNA TSPY (Y-231) showed 97% homology to the human TSPY cDNA pJA923 (1.6 kb), and testis-specific expression. TSPY is present in several copies, and at least three differently sized TSPY transcripts exist, caused by different patterns of splicing (Zhang et al., 1992; Manz et al., 1993). The exact number of transcribed genes or pseudogenes is not yet known. TSPY sequences are organized as constitutive parts of DYZ5 repeat units (Manz et al., 1993) which are located on the short arm of the Y chromosome (Tyler-Smith et al., 1988). Evolutionary conservation of DYZ5 (Y-190) sequences on the Y chromosome of the great apes was suggested by Southern blot and in situ hybridization (Guttenbach et al., 1992). Using the chromosomal in situ hybridization, Schempp et al. (1995) demonstrated that specific members of the YRRM (Y chromosome RNA recognition motif) and TSPY families are evolutionarily conserved and Y chromosome–specific in hominoids.

The aim of this study is to: (1) compare the sequence divergence of TSPY genes between great apes and humans; (2) determine the phylogenetic relationship among human, chimpanzee, gorilla, and orangutan; and (3) deduce the rate of nucleotide substitution per site per year for TSPY gene sequences.

MATERIALS AND METHODS

In hominoids specific portions of the TSPY are evolutionarily conserved (Guttenbach et al., 1992; Schempp et al., 1995). Accordingly, we examined single individuals from different species to compare with the human sequence. Parts of the TSPY genes of chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), and baboon (*Papio hamadryas*) were amplified by the polymerase chain reaction (PCR) using the following primers: A: 5′ AGCCAGGAAGGCCTTTTCTCG 3′; B: 5′ CCATGTAGCTCAGCATGTCTTCAT 3′.

The 5′ of primer A and the 3′ of primer B correspond to nucleotide number 324 and 1,093, respectively, of the human sequence (Zhang et al., 1992). The PCR was performed with a Thermocycler of Perkin Elmer Cetus (Model 480). The standard conditions were 94°C for 1 min, 60°C for 1 min, 72°C for 1.5 min for 30 cycles. This main PCR amplification cycle was preceded by an initial denaturation step of 94°C for 4 min and followed by an additional extension period of 72°C for 10 min. PCR products were cloned into *Sma*I-cut, alkaline phosphatase–treated pUC 118 plasmid vector, transformed, and replicated in *Escherichia coli* K-12 strain, JM 109. Plasmid DNA were extracted by an automatic plasmid isolation system (Pharmacia). Nucleotide sequences were determined on both strands of plasmid DNA using the dideoxy chain termination method (Sanger et al., 1977) with *Taq* polymerase and the M13 universal primers (Pharmacia). At least three cloned fragments from each of the PCR-amplified DNAs were sequenced.

Sequences were aligned with the aid of GENETYX (Version 9, SDC Tokyo), and sequence divergence was calculated for all pairwise comparisons following the Jukes and Cantor method (1969). The phylogenetic trees were constructed by the neighbor-joining (NJ) (Saitou and Nei, 1987), maximum likelihood (ML), and maximum parsimony (MP) methods of the PHYLIP3.5c (Felsenstein, 1993).

RESULTS

Pairwise comparisons of TSPY gene sequences

We sequenced an approximately 755 bp region of the TSPY gene including part of exon 1, exon 2, and the first intron for chimpanzee (*Pan troglodytes*), gorilla (*Gorilla gorilla*), orangutan (*Pongo pygmaeus*), and baboon (*Papio hamadryas*) as an outgroup (Fig. 1). The length of the aligned sequence corresponds to the homologous region of bp 346–1,093 in the human TSPY gene sequence

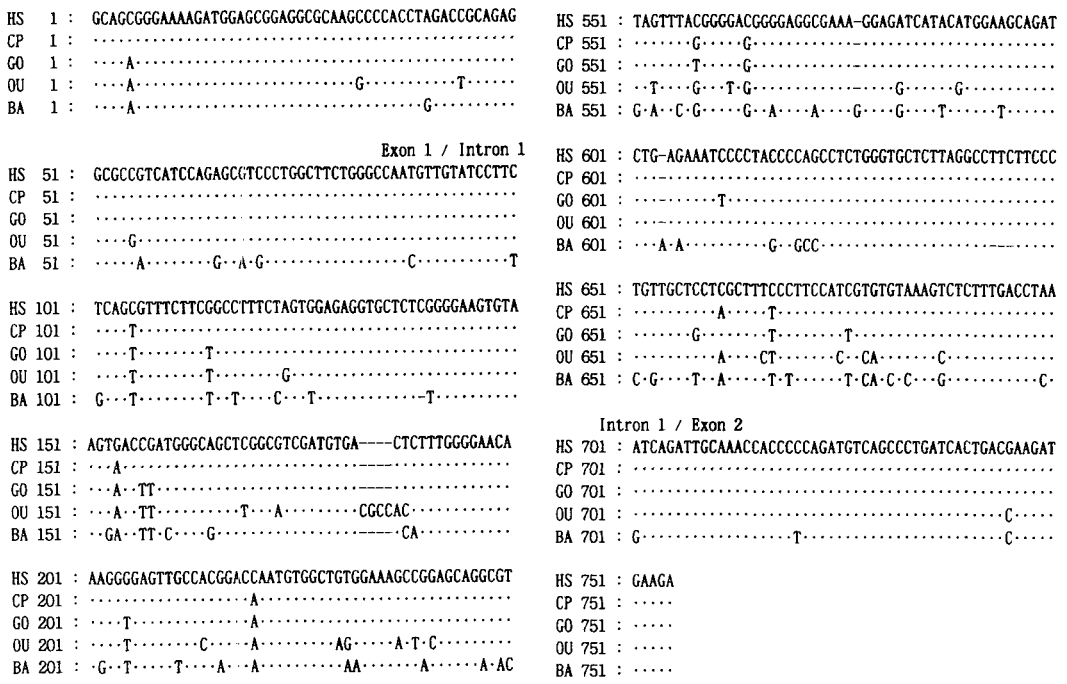

Fig. 1. Sequence alignments including part of exon 1, exon 2, and the first intron of the TSPY gene. Species names are abbreviated as follows. HS, human (*Homo sapiens*); CP, chimpanzee (*Pan troglodytes*); GO, gorilla (*Gorilla gorilla*); OU, orangutan (*Pongo pygmaeus*); BA, baboon (*Papio hamadryas*). Dots mean same as the nucleotide in the top row, and dash means deletion.

(Zhang et al., 1992). The TSPY gene has diverged at 144 of the 755 nucleotide positions. In overviewing five sequences, one deletion in human, four successive nucleotide insertions in orangutan, and seven deletions/insertions in baboon were noted. The substitutions, deletions, and insertions observed in pairwise comparisons among the species are presented in Table 1. Estimates of percent divergence revealed that the TSPY of human and apes shared a high degree of sequence identity. The percent sequences differed from that of human by 1.9% (chimpanzee), 4.0% (gorilla), 8.2% (orangutan), and 16.8% (baboon), respectively. Humans and chimpanzees shared the fewest differences whereas chimpanzees and gorilla differed by 2.7%. On average, species of human and apes varied from baboon by 15.9% (15.0–16.8).

Phylogenetic relationships

The number of nucleotide substitutions for a given pair of species was calculated by the Jukes and Cantor method (1969). Using sequence divergence estimates (Table 1) derived from the TSPY gene, we constructed a phylogenetic tree by the neighbor-joining (NJ) method (Fig. 2a). A similar phylogenetic

TABLE 1. Pairwise divergences among TSPY gene sequences of primates

Species pair	BP[3]	Divergence(%) Uncorrected	Corrected[1]	Gaps[2]
Human–chimp	748	2.0	1.9	1
Human–gorilla	748	4.0	4.0	1
Human–orang	752	8.4	8.2	5
Human–baboon	751	16.0	16.8	8
Chimp–gorilla	748	2.7	2.7	0
Chimp–orang	752	6.6	6.4	4
Chimp–baboon	751	14.5	15.1	7
Gorilla–orang	752	6.5	6.3	4
Gorilla–baboon	751	14.4	15.0	7
Orang–baboon	755	16.0	16.8	11

[1]Jukes and Cantor, 1969.
[2]Gaps refer to both insertion and deletion events.
[3]BP means base pairs under comparison.

tree (Fig. 2b) was also obtained by using the maximum likelihood (ML) method. In addition, these relationships were significantly supported by bootstrap analysis. The strict consensus maximum parsimony tree generated from 100 bootstrap replicates is shown in Figure 2c. With maximum parsimony, eight inferred synapomorphies define a human/chimpanzee clade (at positions 5, 114, 157, 158, 205, 261, 362, and 398). The next most parsimony trees are longer by five events with a human/gorilla clade (at positions 188, 268, 582, 679, and 745). When transitions, transversions, or both are used, trees support a human/chimpanzee clade. In relative internodes for the human-chimpanzee-gorilla trichotomy among molecular phylogenies, the TSPY gene shows a long intercode. The clade which contains human, chimpanzee, and gorilla was significantly supported at the 95.8% level. The human and chimpanzee relationships were supported with bootstrap probabilities of 99%. Thus, the topology obtained by neighbor-joining and maximum likelihood analysis as identical to the maximum parsimony tree.

Rate of nucleotide substitutions

We have calculated the number of nucleotide substitutions per site (KN) using the method of Jukes and Cantor (1969). As shown in Table 2, KN values for the TSPY intron between human and the present sequence of chimpanzee, gorilla, orangutan, baboon were 0.024, 0.048, 0.094, and 0.196, while those values for the TSPY exon were 0, 0.007, 0.036, and 0.067, respectively. KN values for the TSPY intron were higher than that for the zinc finger Y (ZFY) intron (Dorit et al., 1995). The highest KN value was found in human–baboon comparison of the TSPY intron. The number of nucleotide substitutions was not uniform among genes.

Using these KN values, we estimated the rate of nucleotide substitutions per site per year, and based divergence time on the paleontological data as shown in Table 3 (Delson, 1980; Pilbeam, 1984; Andrews, 1986). In the TSPY intron, the rate was: (1) 1.2–2.4 $\times$ 10^{-9}/site/year between human and chimpanzee, (2) 2.0–3.4 $\times$ 10^{-9}/site/year between human and gorilla, (3) 2.6–3.6 $\times$ 10^{-9}/site/year between human and orangutan, and (4) 3.3–4.9 $\times$ 10^{-9}/site/year between human and baboon. The rates of nucleotide substitutions of human–gorilla and human–orangutan were 0.3–0.5 $\times$ 10^{-9}/site/year and 1.0–1.4 $\times$ 10^{-9}/site/year for the TSPY exon, respectively. Rates of nucleotide substitutions per site per year were higher in the TSPY intron than in the TSPY exon, and higher in the TSPY intron than in the ZFY intron in human and apes.

DISCUSSION

The percentage divergence estimates calculated for higher primates clearly support the contention that humans and great apes share a high degree of sequence identity (Table 1). The aligned DNA sequences of hu-

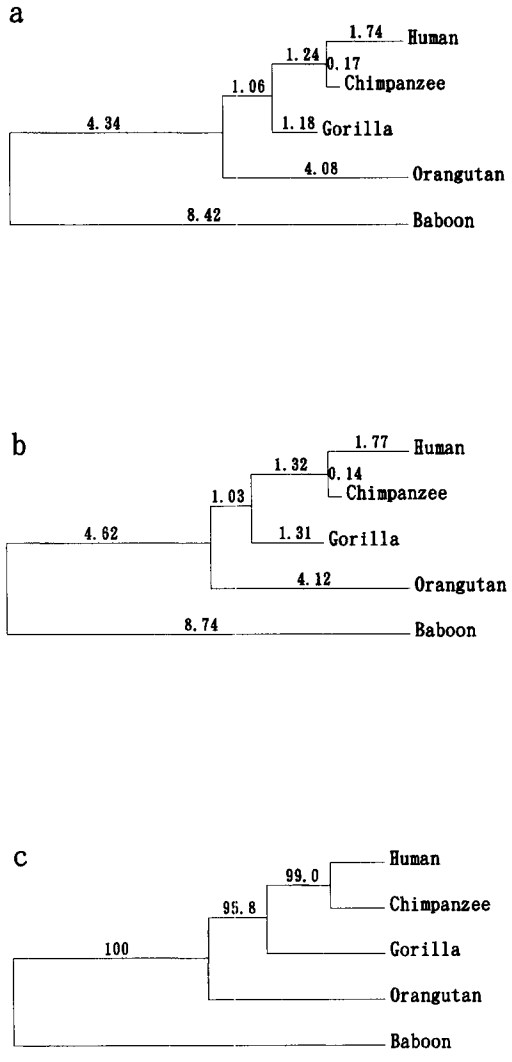

Fig. 2. Phylogenetic trees obtained by **(a)** the neighbor-joining (NJ), **(b)** maximum likelihood (ML), and **(c)** maximum parsimony (MP) methods for the hominoid primates. The baboon was the designated outgroup in this analysis. Divergence distances in the NJ and JL trees are written above the branches. Bootstrap values of the consensus MP tree (node reproduction frequencies out of 100 trees) are represented from bootstrap analysis. These three methods considered only nucleotide substitutions, and ignored insertions and deletions.

man, chimpanzee (*Pan troglodytes*), and gorilla varied by 1.9–4.0%, with human and chimpanzee being most alike. On average, the nucleotide sequence of orangutan differed from the other three (humans and African apes) by 7.25%. These values conform closely to the divergence estimates calculated from PABY (Table 4). Overall, human and apes differ by 2.4–9.4% according to our results. These measurements were more similar with PABY than pseudoautosomal boundary X (PABX), β-globin noncoding, and DNA–DNA hybridization. The smallest interspecific divergence of the TSPY intron (2.4% for human and chimpanzee) was threefold lower than that of the mitochondrial ND4 and ND5 gene (Hayasaka et al., 1988) and approximately 1.5-fold lower than that of the mitochondrial 12S rRNA gene (Hixon and Brown, 1986). In the nuclear genomic regions, the divergence of TSPY intron for human and chimpanzee was similar to 28S rRNA (ITS1) (Gonzalez et al., 1990). In comparison of relative internodes for the human-chimpanzee-gorilla trichotomy among molecular phylogenies, TSPY gene sequences are 2.5-fold longer than PABY (Ellis et al., 1990) and DNA–DNA hybridization data (Caccone and Powell, 1989), and fourfold longer than mitochondrial cytochrome oxidase subunit II (Ruvolo et al., 1991).

The sequence divergence of the Y-chromosomal DNA among related genera and species can be useful in refining evolutionary classification, and Y-chromosomal DNA sequences represent a valuable new source of characters for phylogenetic inference (Rasheed et al., 1991; Lundrigan and Tucker, 1994). We constructed three phylogenetic trees (NJ, ML, and MP) using testis-specific gene sequences (TSPY), and all support the suggestion that the chimpanzee is the closest extant relative to humans. The same conclusion was drawn in other molecular studies where relatively small numbers of nucleotide differences were found. Gonzalez et al. (1990) compared sequences totaling 3.5 kb from the 28S rRNA gene and an internal transcribed spacer (ITS) region for the human, chimpanzee, gorilla, and orangutan. Based on distance values, they determined that human and chimpanzee were the most related pair. Bailey et al. (1992) and Goodman et al. (1993) addressed the trichotomy issue by using maximum parsimony and maximum likelihood analysis on a data set of orthologous noncoding sequences repre-

TABLE 2. Mean ± standard error of the number of nucleotide substitutions per site in TSPY and ZFY genes

Species pair	TSPY		ZFY[1]
	Exon (141 bp)	Intron (610 bp)	Intron (729 bp)
Human–chimp	0 ± 0	0.024 ± 0.006	0.007 ± 0.003
Human–gorilla	0.007 ± 0.007	0.048 ± 0.009	0.014 ± 0.004
Human–orang	0.036 ± 0.016	0.094 ± 0.013	0.043 ± 0.008
Human–baboon	0.067 ± 0.023	0.196 ± 0.020	—
Chimp–gorilla	0.007 ± 0.007	0.032 ± 0.007	0.015 ± 0.005
Chimp–orang	0.036 ± 0.016	0.071 ± 0.011	0.044 ± 0.008
Chimp–baboon	0.067 ± 0.023	0.174 ± 0.019	—
Gorilla–orang	0.029 ± 0.015	0.071 ± 0.011	0.045 ± 0.008
Gorilla–baboon	0.059 ± 0.021	0.174 ± 0.019	—
Orang–baboon	0.075 ± 0.024	0.193 ± 0.020	—

[1] Dorit et al., 1995.

senting up to 8.3 kb of the γ-globin gene and 12.5 kb of the ψη-globin pseudogene sequence. Both parsimony and likelihood methods yielded the same branching pattern, which indicated that human and chimpanzee share the most recent common ancestry. This phylogenetic tree is also supported by results from DNA–DNA hybridization (Sibley and Ahlquist, 1987; Caccone and Powell, 1989). Using the Y chromosome pseudoautosomal region sequences, the most parsimonious tree has human and chimpanzee branching after the divergence of gorilla, whereas in the X chromosome pseudoautosomal region sequences, the most parsimonious tree has chimpanzee and gorilla branching after the divergence of human (Ellis et al., 1990).

In order to estimate the substitution rate between two species, we have to know their divergence time. However, divergence times are usually not well established, so we were restricted to the paleontological data for every pair of species compared (Delson, 1980; Pilbeam, 1984; Andrews, 1986). In the comparison between human and chimpanzee, the rate of nucleotide substitution varies considerably among the genes studied (Table 3). This is probably due largely to chance effects, because the number of nucleotide sites in each gene is small and the degree of sequence divergence is low. The rate of nucleotide substitution per site per year (Vn) of the TSPY intron was almost threefold higher than the ZFY intron in human–chimpanzee and human–gorilla (Dorit et al., 1995). In comparing the TSPY intron and other genes (human–orangutan), Vn of TSPY intron was twofold higher than the ZFY intron (Dorit et al., 1995), threefold higher than the sex-determining region Y (SRY) exon (Whitfield et al., 1993), and 1.5-fold higher than the Alu-distal of PABY (Ellis et al., 1990). Vn of SRY exon and Alu-proximal of PABY was identical at 0.5–0.9 $\times 10^{-9}$/site/year in human–gorilla, and Vn of SRY exon (1.1–1.6 $\times 10^{-9}$/site/year) was almost similar to TSPY exon in human–baboon (Table 3). The rate of nucleotide substitution of the TSPY intron was higher than the ZFY intron and PABY.

In summary, we have sequenced a part of exon 1, exon 2, and the first intron, a total of 3,020 bp, of the TSPY gene in chimpanzee, gorilla, orangutan, and baboon. The sequence divergences were more similar with PABY than PABX, β-globin noncoding, or DNA–DNA hybridization. Phylogenetic trees calculated by three different methods (neighbor-joining, maximum likelihood, and maximum parsimony) suggest that human and chimpanzee are more closely related to each other than either of them is to gorilla. The rates of nucleotide substitutions per site per year were higher in the TSPY intron than either the TSPY exon or the ZFY intron in human and apes.

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TABLE 3. Rates of nucleotide substitution per site per year in primates

Species pair	Divergence[1] time (10^6 yr)	$K_N \times 100$[5]						Rate ($\times 10^{-9}$)					
		PABY[2]		SRY[3]	ZFY[4]	TSPY		PABY		SRY	ZFY	TSPY	
		Alu-D	Alu-P	Exon	Intron	Exon	Intron	Alu-D	Alu-P	Exon	Intron	Exon	Intron
Human–chimp	(5–10)	0	1.6	1.3	0.7	0	2.4	0	(0.8–1.6)	(0.7–1.3)	(0.4–0.7)	0	(1.2–2.4)
Human–gorilla	(7–12)	3.4	1.2	1.2	1.4	0.7	4.8	(1.4–2.4)	(0.5–0.9)	(0.5–0.9)	(0.6–1.0)	(0.3–0.5)	(2.0–3.4)
Human–orang	(13–18)	6.0	5.3	2.7	4.3	3.6	9.4	(1.7–2.3)	(1.5–2.0)	(0.8–1.0)	(1.2–1.7)	(1.0–1.4)	(2.6–3.6)
Human–baboon	(20–30)	10.3	12.6	6.5	—	6.7	19.6	(1.7–2.6)	(2.1–3.2)	(1.1–1.6)	—	(1.1–1.7)	(2.5–4.9)

[1] Delson, 1980; Pilbeam, 1984; Andrews, 1986.
[2] Ellis et al., 1990.
[3] Whitfield et al., 1993.
[4] Dorit et al., 1995.
[5] K_N means the number of nucleotide substitutions per site.

TABLE 4. Comparison of sequence divergence as estimated from PABY/PABX, TSPY, β-globin sequences, and from DNA hybridization

Species pair	Divergence (%)			
	PABY/PABX[1]	TSPY intron (this study)	β-globin cluster[2] noncoding	Genomic DNA–DNA[3] hybridization ($T_{50}H$)
Human–chimp	1.5/1.3	2.4	1.7	1.6
Human–gorilla	2.9/2.7	4.8	1.8	2.3
Human–orang	7.8/3.4	9.4	3.3	3.6
Human–baboon	10.7/8.6	19.6	—	7.3
Chimp–gorilla	3.4/1.0	3.2	1.7	2.3
Chimp–orang	7.8/3.4	7.1	3.5	3.6
Chimp–baboon	10.7/8.3	17.4	—	7.2
Gorilla–orang	8.7/3.4	7.1	3.5	3.6
Gorilla–baboon	11.1/8.6	17.4	—	7.2
Orang–baboon	9.7/8.3	19.3	—	7.4

[1] Ellis et al., 1990.
[2] Goodman et al., 1990.
[3] Sibley and Ahlquist, 1987.

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